JCI The Journal of Clinical Investigation

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J Clin Invest. 2021. https://doi.org/10.1172/JCI144847.

Clinical Medicine In-Press Preview Endocrinology Oncology

Graphical abstract

NTRK- and RET-fusion-directed therapy in pediatric thyroid cancer yields a tumor response and radioiodine uptake

Short title: Fusion oncogenes in pediatric thyroid cancer

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

Role of the funding source

The study was designed by the authors. Two patients participated in a clinical trial and received fusion-targeted therapy. All this research except the clinical trials was supported by the Ministry of Science, ICT & Future Planning (grant number NRF-2016R1A2B4012417 and 2019R1A2C2084332), the Ministry of Health & Welfare, Republic of Korea (grant number H14C1277), the Ministry of Education (grant number 2020R1A6A1A03047972), and the Seoul National University Hospital Research Fund (grant number 04-2015-0830). The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Abstract

Background: Molecular characterization in pediatric papillary thyroid cancer (PTC), distinct from adult PTC, is important for developing molecular targeted therapies for progressive ^{131}I-refractory PTC.

Methods: PTC samples from 106 pediatric patients (age: 4.3–19.8 years; 22 boys) who attended Seoul National University Hospital (January 1983–March 2020) were available for genomic profiling. Previous transcriptome data from 125 adult PTCs were used for comparison.

Results: Genetic drivers were found in 80 tumors; 31 with fusion oncogenes (*RET* in 21, *ALK* in 6, and *NTRK1/3* in 4), 47 with point mutations ($BRAF^{V600E}$ in 41, $TERT^{C228T}$ in 2, and *DICER1* variants in 5), and 2 with amplifications. Fusion-oncogene PTCs, predominantly detected in younger patients, presented with a more advanced stage and showed more recurrent or persistent disease than $BRAF^{V600E}$ PTCs, which were detected mostly in adolescents. Pediatric fusion PTCs (in those aged < 10 years) showed lower expression of thyroid differentiation genes, including *SLC5A5*, than adult fusion PTCs. Two girls with progressive ^{131}I-refractory lung metastases harboring a *TPR-NTRK1* or *CCDC6-RET* fusion received fusion-targeted therapy; larotrectinib and selpercatinib decreased the tumor extent and restored radioiodine uptake. The girl with the *CCDC6-RET* fusion received ^{131}I therapy combined with selpercatinib, leading to a tumor response. *In vitro* ^{125}I uptake and ^{131}I clonogenic assays showed that larotrectinib inhibited growth and restored radioiodine avidity.

Conclusions: In pediatric fusion-oncogene PTC cases with ^{131}I-refractory advanced disease, selective fusion-directed therapy may restore radioiodine avidity and lead to a dramatic tumor response, underscoring the importance of molecular testing in pediatric PTC patients.

Funding: The Ministry of Science, ICT & Future Planning (grant number NRF-2016R1A2B4012417 and 2019R1A2C2084332), the Ministry of Health & Welfare, Republic of Korea (grant number H14C1277), the Ministry of Education (grant number 2020R1A6A1A03047972), and the Seoul National University Hospital Research Fund (grant number 04-2015-0830).

Introduction

Pediatric papillary thyroid cancers (PTCs) have distinct genetic alterations, with a higher proportion of gene fusions compared to adult PTCs, in which point mutations predominate (1).

Previous methods to detect *BRAF*^{V600E}, *RAS*, or *RET/PTC* gene alterations identified driver alterations in less than half of pediatric PTCs (2). However, next-generation sequencing (NGS) has increased the detection rate of genetic drivers in sporadic cases by more than 60% (3-7). A significant proportion of fusion oncogenes has been detected (20–60%), similar to radiation-associated thyroid cancers (8). This high frequency of oncogenic fusion may be important in the pathogenesis of pediatric PTCs, and could facilitate the selection of patients for recently developed, highly selective and potent fusion-targeted agents (9, 10). Moreover, identifying transcriptomic characteristics may help determine the pathogenesis and biological behavior of pediatric PTCs and differentiate them from adult PTCs. Previous molecular studies of pediatric PTCs have generally been too small to generate age-associated genomic profiles, and transcriptomic information on these tumors is limited.

In this study, we comprehensively characterized age-associated genetic alterations in a large series of pediatric PTCs. Furthermore, we performed transcriptome analyses of pediatric PTCs and compared these profiles with those reported for adult PTCs (11). Based on these data, we investigated the role of the *NTRK* and *RET* fusion-targeted agents, larotrectinib and selpercatinib, respectively, in the inhibition of tumor growth and restoration of radioiodine uptake in progressive ^{131}I-refractory PTCs in young children.

Results

Age-associated genetic alterations in pediatric PTCs

The clinicopathological characteristics and genetic analyses of 106 Korean patients (age range: 4.3–19.8 years; 22 boys and 84 girls) are summarized in Table 1 and Figure 1. Genomic alterations were found in 80 patients, including 31 with oncogenic fusions (*NTRK1/3* in 4, *RET* in 21, and *ALK* in 6), 47 with point mutations (*BRAF*^{V600E} in 41, *TERT*^{C228T} in 2 [1 of whom had a coexisting *BRAF*^{V600E}], and *DICER1* variants in 5), and 2 with *FGFR1* or *EGFR* amplifications (Supplemental Tables 1 and 2). Detailed information on the fusion partner genes, breakpoints, and methods to detect each fusion oncogene are described in Table 2, Supplemental Table 3, and Supplemental Figure 1. *H/K/NRAS* mutations were not identified in any of the tested tumors. Among nine patients who underwent radiotherapy, a genetic driver was identified in seven (2 *RET*, 1 *ALK*, 1 *BRAF*^{V600E}, 1 *DICER1*$^{p.D1709G}$ with coexisting loss of heterozygosity at multiple chromosomal loci, and two amplifications; Supplemental Table 1).

In the < 10 (n = 14), 10–14 (n = 40), and 15–19 years (n = 52) age groups, the proportions of fusion oncogenes were 92.9%, 27.5% and 13.5%, while the point mutation rates were 7.1%, 30.0% and 65.4% (Figure 2A), with *BRAF*^{V600E} rates of 0%, 27.3% and 57.7%, respectively. The frequency of each gene according to age is shown in Figure 2B and Supplemental Table 2. In particular, among 14 young children aged < 10 years, 13 harbored a fusion oncogene (9 *RET*, 2 *NTRK*, and 2 *ALK*) and 1 had a *TERT*^{C228T} mutation. The pooled analysis (Supplemental Table 4) demonstrated similar trends between the < 10 and 10–22 years age groups (Figure 2C and 2D).

Clinicopathological characteristics and outcomes according to the genetic alterations in pediatric PTCs

Oncogenic fusion PTCs were associated with a higher proportion of large tumors (> 2 cm), extrathyroidal extension, and lymph node (LN) and lung metastasis compared to *BRAF*^{V600E} PTCs (Tables 1 and 2, and Supplemental Table 5). *RET* fusion PTC was predominantly a diffuse-sclerosing variant (DSV, 55.0%), while *BRAF*^{V600E} PTC was mostly a classic variant (89.7%). Oncogenic fusion

PTCs were categorized into two age groups [fusion < 10 years (n = 13), and fusion 10–19 years (n = 18) groups], and *BRAF*^{V600E} PTCs were all in the 10–19 years group (n = 41). The proportion of large tumors, extrathyroidal extension, LN, lung metastasis (Figure 2E), and biochemical or structural disease (Figure 2F) significantly decreased from the fusion < 10 years group to the fusion 10–19 and *BRAF*^{V600E} 10–19 years groups. Among the 13 patients with persistent lung metastasis despite ^{131}I treatment (2 *NTRK1*, 7 *RET*, 2 *ALK*, 1 *DICER1*, and 1 with no driver identified), 10 maintained stable status, while 3 young children (P1 with a *TPR-NTRK1*, P8 with a *CCDC6-RET*, and P11 with an *ERC1-RET* fusion) had ^{131}I-refractory progressive disease (Table 2). The former 2 girls (P1 and P8) were exposed to the fusion-targeted kinase inhibitor described below. The other case, a 9-year-old boy (P11) with an *ERC1-RET* fusion, showed mixed responses resulting in a progressively decreased uptake of radioactive iodine during repeated high-dose ^{131}I therapy (cumulative dose = 520 mCi/5 times) (Supplemental Figure 2). He is currently planning to participate in a phase III clinical trial of fusion-targeted therapy.

Comparison of clinicopathological characteristics and outcomes between pediatric and adult PTCs

We compared the clinicopathological presentation and outcomes between pediatric and adult patients at Seoul National University Hospital (11) according to whether the genetic driver was a fusion oncogene (*NTRK1/3*, *RET*, or *ALK*) or *BRAF*^{V600E}. The pediatric fusion group (n = 31) exhibited higher rates of extrathyroidal extension (Figure 2G) and biochemical or structural disease (Figure 2H) than the adult fusion group (n = 12). Although the adult *BRAF*^{V600E} group (n = 68) had a higher rate of LN metastasis than the pediatric *BRAF*^{V600E} group (n = 41), disease outcomes did not differ between the pediatric and adult *BRAF*^{V600E} groups (Supplemental Table 6, Figure 2G and H). When our pediatric data were compared to The Cancer Genome Atlas (TCGA) database, including the adult fusion group (n = 42) and *BRAF*^{V600E} group (n = 241), similar results were obtained (Supplemental Table 7).

Comparison of gene expression between pediatric and adult PTCs

A more advanced presentation and worse outcome of oncogenic fusion PTCs, and their predominance among younger patients, imply distinct molecular characteristics that differ between pediatric and adult PTCs (12). The gene expression profiles of nine oncogenic fusion PTCs from children clustered closer to those of the adult *BRAF*-like group, while adult PTCs harboring fusions were scattered (Figure 3A) (11). Figure 3B shows age-associated clustering of oncogenic fusion PTCs. As indicated in Figure 3C and Supplemental Figure 3, pediatric oncogenic fusion PTC cases, in particular in the < 10 years age group, showed higher expression of mitogen-activated protein kinase (MAPK) signaling pathway genes (ERK score), and lower expression of genes related to thyroid differentiation (thyroid differentiation score [TDS]), than the adult fusion PTC groups (11, 13). Higher ERK scores and lower TDS were also found in the pediatric *BRAF* group compared to the adult *BRAF* group (Figure 3C). The transcriptomic expression analysis of individual TDS genes demonstrated that several genes, including *SLC5A5, SLC26A4, SLC5A8, DIO1,* and *DIO2*, tended to have lower expression levels in pediatric fusion PTCs (< 10 years) compared to adult fusion PTCs (Figure 3D). Notably, the expression of *SLC5A5* (sodium-iodide symporter, NIS), which is an important determinant of ^{131}I avidity, also decreased in childhood-fusion PTCs. However, the difference was not significant due to the limited number of fresh tissues; therefore, we also explored the lower TDS and lower expression of the *SLC5A5* gene in pediatric fusion tumors compared to normal tissues by analyzing the formalin-fixed paraffin-embedded (FFPE) samples of eight fusion PTCs from young children (Figure 3E). Remarkably, the two ^{131}I-refractory progressive PTC cases exhibited very low expression of *SLC5A5* in their tumor tissues (P1 in Figure 3D, and P8 in Figure 3E).

Larotrectinib decreases the tumor extent and restores radioiodine uptake in ^{131}I-refractory progressive metastatic TPR-NTRK1 fusion-positive pediatric PTCs

A 4.3-year-old girl (P1 in Table 2) was diagnosed with a 3.6-cm classic variant PTC with extensive LN involvement and lung metastases. She underwent total thyroidectomy and neck dissection, followed by the administration of 30 mCi (0.06 GBq/kg) ^{131}I. The post-treatment whole-body scan (WBS) revealed

remnant thyroid uptake only (Figure 4A, upper left). No ^{131}I uptake was identified on the post-treatment scan after the second dose of 30 mCi (Figure 4A, upper right), despite locoregional recurrence and progressive lung disease (Figure 4B, baseline). The thyrotropin (TSH)-stimulated serum thyroglobulin level was 1,150 ng/mL. A *TPR-NTRK1* rearrangement was identified. Larotrectinib was initiated at 100 mg orally, twice daily (NCT02576431; NAVIGATE). Computed tomography (CT) revealed a dramatic improvement in the LN and lung metastases after 4 weeks (Figure 4B), and a complete response at 21 months, according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1. After 12 weeks of therapy, radioiodine uptake was shown to be restored in the neck and lungs by a diagnostic ^{123}I scan (Figure 4A, lower left and right). She did not undergo ^{131}I therapy due to participation in the clinical trial and has remained in response without dose-limiting toxicity at 41 months (Figure 4B).

Selpercatinib decreases tumor extent and restores radioiodine uptake in ^{131}I-refractory progressive metastatic CCDC6-RET fusion-positive pediatric PTC

A 7.4-year-old girl (P8 in Table 2) was diagnosed with a 2.8-cm DSV PTC with LN involvement and lung metastases. She underwent total thyroidectomy and neck dissection, followed by the administration of 50 mCi (0.11 GBq/kg) ^{131}I. The post-treatment WBS identified minimal lung uptake (Figure 4C, left). The TSH-stimulated serum thyroglobulin level was 5,990 ng/mL. After 4 months, locoregional recurrence and progressive lung metastasis were detected (Figure 4D, baseline). A *CCDC6-RET* rearrangement was identified. Selpercatinib was initiated at 80 mg orally, twice daily (#LOXO-RET-18018). The lung lesions were markedly decreased in extent according to a chest radiograph at 10 days (Figure 4D, upper right). Since achieving a partial response after 4 weeks according to RECIST v1.1 (Figure 4D, lower middle), she has remained in response with no dose-limiting toxicity. Radioiodine uptake was restored in the lung on a diagnostic ^{123}I scan at 5 months (Figure 4C, middle), which enabled administration of 60 mCi (0.11GBq/kg) ^{131}I combined with selpercatinib, leading to remarkable radioiodine uptake in the entire lung field at 13 months (Figure 4C, right) and a TSH-stimulated serum thyroglobulin level of 1,930 ng/mL. ^{131}I therapy of 60 mCi (0.11GBq/kg) was additionally administered

after 19 months of the selpercatinib therapy, leading to persistent radioiodine uptake in the lung field with a TSH-stimulated serum thyroglobulin level of 855 ng/mL. CT revealed stable lung disease at 29 months (Figure 4D, lower right).

In vitro effects of larotrectinib on tumor growth and radioiodine uptake capacity

The restoration of radioiodine uptake in ^{131}I non-avid lesions after larotrectinib and selpercatinib treatment implies that these selective inhibitors not only abrogate cellular proliferation but also induce restoration of iodine uptake and processing in these cancers, similar to previous reports of MAPK inhibitors (14-16).

In vitro experiments showed that basal ^{125}I uptake was markedly decreased in $Nthy^{TPR\text{-}NTRK}$ cells compared to control $Nthy^{WT}$ cells, but was restored by larotrectinib treatment (Figure 5A, Supplemental Figure 4A and 4B). This larotrectinib-induced restoration was mediated by NIS, as indicated by the effects being blocked by potassium perchlorate ($KClO_4$), a competitive inhibitor of iodide transport through the NIS (Figure 5A, Supplemental Figure 4C). A trend toward increased expression of the NIS at the mRNA and protein levels was associated with larotrectinib treatment in $Nthy^{TPR\text{-}NTRK}$ cells (Figure 5B and 5C, Supplemental Figure 4D), but not in $Nthy^{WT}$ cells (Supplemental Figure 4D). To evaluate whether larotrectinib treatment enhances the therapeutic effect of ^{131}I, $Nthy^{TPR\text{-}NTRK}$ cells were pre-treated with larotrectinib followed by 100 μCi of ^{131}I. While ^{131}I alone did not suppress the colony-forming ability in $Nthy^{TPR\text{-}NTRK}$ cells, larotrectinib alone inhibited colony formation. Moreover, the combination of ^{131}I and larotrectinib further enhanced the inhibition of colony-forming (Figure 5D, Supplemental Figure 4E).

Discussion

Our comprehensive genomic analysis revealed age-associated driver profiles of pediatric PTC. Oncogenic fusions predominated in children aged < 10 years with PTCs, after which the frequency decreased to levels similar to those seen in adults. Furthermore, the incidence of driver-point mutations increased with age, and became common in adolescents aged 15–19 years, as in adults. Pediatric oncogenic fusion PTCs presented with more advanced disease and had worse outcomes than *BRAF*^{V600E} PTCs. The transcriptomic data showed that pediatric oncogenic fusion PTCs in young children less than 10 years of age had a lower TDS (including NIS expression) than adult fusion PTCs. *NTRK* and *RET* fusion-targeted therapy with larotrectinib or selpercatinib yielded a remarkable tumor response, and restored radioiodine uptake in two pediatric patients with ^{131}I-refractory progressive PTCs harboring *TPR-NTRK1* and C*CDC6-RET* fusion, respectively.

The detection rate of genetic alterations was 75.5% in our pediatric PTC population with the use of NGS, fluorescence *in situ* hybridization (FISH), and immunohistochemistry (IHC). This was the largest pediatric study to date showing age-associated genetic alterations, consistent with a pooled analysis of previously reported cases, including a large recent pediatric study of 93 patients that used DNA and RNA sequencing (Supplemental Table 4) (2, 17). Oncogenic fusions accounted for the majority of cases among children aged < 10 years, while *BRAF*^{V600E} was the most common driver in adolescents, with a frequency similar to that seen in adults (13, 18). *DICER1* was the second most common point mutation, consistent with a recent pediatric study (19). However, *TERT* promoter and *RAS* mutations were uncommon, in line with previous pediatric reports (Supplemental Table 4) (2-7, 17, 20-22).

The etiology of age-associated genetic alterations remains unexplained, although chromosomal rearrangements have a strong association with exposure to ionizing radiation, while *BRAF*^{V600E} point mutations may be linked to excess dietary iodine intake or exposure to chemical elements in volcanic areas (23). DNA fragility and repair defects have been suggested as mechanisms for radiation-induced

genetic changes or spontaneous oncogenic fusion (4, 5, 24). The thyroid cells of young children may be more susceptible to the effects of ionizing radiation and/or lose key factors in the DNA repair machinery, leading to uncoupled double-strand breaks and translocation with partner genes (24). Analysis of post-Analysis of post-Chernobyl thyroid cancers showed that the mean age at radiation exposure was lower in patients with tumors harboring oncogenic fusion genes (7.1 years) than in those with tumors harboring point mutations (10.9 years) (25). As almost all sporadic PTC cases aged < 10 years also harbored oncogenic fusion in this study, further study to determine as yet unknown risk factors for the development of fusions is needed.

Children with oncogenic fusion PTCs presented with more advanced stage disease; 42% of the children had lung metastasis, and a higher risk for recurrence or persistence than those with $BRAF^{V600E}$ PTC. In particular, among 13 cases with persistent lung metastasis, the disease was stable in 10 patients, while it progressed in 3, despite ^{131}I therapy. Consistent with previous reports (4, 21), the lower TDS and higher ERK scores demonstrate the aggressiveness of oncogenic fusion PTCs in young children. Although the influence of the $BRAF^{V600E}$ mutation alone on tumor aggressiveness remains controversial (21), synergistic effects of $TERT^{C228T/C250T}$ and $BRAF^{V600E}$ mutations have been shown to lead to a worse prognosis in patients with PTC (26). Therefore, the very low frequency of $TERT^{C228T/C250T}$ mutations in pediatric PTCs (2, 3, 5, 7, 17, 20) may explain the less aggressive behavior of pediatric $BRAF^{V600E}$ PTCs (21). The reason for the more aggressive nature of pediatric oncogenic fusion PTCs remains unclear.

The low expression of *SLC5A5* (NIS) could explain the radioiodine refractoriness of these tumors. Similar downregulation of thyroid differentiation genes, including *SLC5A5*, has been reported in cases of post-Chernobyl oncogenic fusion PTC (8), although the reported effects of oncogenic fusions on thyroid cancer dedifferentiation are inconsistent (27). It is important to elucidate the genetic alterations and corresponding targeted drugs that most affect the response to ^{131}I therapy depending on NIS expression. In this study, two young girls with ^{131}I-refractory progressive PTC and markedly decreased

NIS expression exhibited dramatic responses to oncogenic fusion-targeted therapy, which not only decreased tumor size but also restored radioiodine uptake.

The tumor responses in this study were consistent with previous reports of TRK fusion-positive thyroid cancer patients treated with larotrectinib (9, 28), and a recent report of *RET*-altered medullary thyroid cancer patients treated with selpercatinib (29). Surprisingly, however, the combination of selpercatinib and ^{131}I therapy enhanced radioiodine uptake and yielded a remarkable tumor response in a girl with PTC harboring a C*CDC6-RET* fusion, implying that selpercatinib could be an effective re-differentiation therapy in ^{131}I-refractory advanced tumors harboring the *RET* fusion oncogene. The treatment response decreased after the third ^{131}I treatment in a 9-year-old boy (P11) recruited to a clinical trial of fusion-targeted therapy. Assuming restoration of ^{131}I-avidity in the girl harboring the *RET* fusion oncogene, it would have been helpful if the 9-year-old boy had received selpercatinib in combination with the third ^{131}I treatment. *In vitro* experiments also support the efficacy of larotrectinib for restoring NIS expression and radioiodine avidity, in addition to inhibiting tumor growth. Therefore, this study supports further investigation of fusion-targeted therapy for redifferentiation of ^{131}I-refractory progressive thyroid cancer (14-16). Our pediatric cases are in agreement with a recent adult case report on larotrectinib-enhanced ^{131}I uptake in advanced PTC (30). Considering the predominance of oncogenic fusions in pediatric PTC patients, and their association with tumor aggressiveness, recently developed, potent and specific kinase inhibitors targeting oncogenic fusions in PTC could be the optimal therapeutic option for ^{131}I-refractory advanced PTCs in children. Furthermore, reactivation of iodine uptake indicates that retreatment with ^{131}I can be considered in previous ^{131}I-refractory PTC patients receiving fusion-targeted therapy (30). Targeted oncogene therapies before surgery may induce tumor regression in cases of invasive thyroid cancer (31). Diagnostic molecular testing to detect driver oncogenic fusions and point mutations is becoming imperative in such cases.

This study was limited by the small number of fresh pediatric tissue samples, so the difference in gene expression between pediatric and adult PTCs needs to be further replicated in a large-sample study. To date, there are no available published data on pediatric PTCs allowing comparison of gene expression with adult PTCs. In addition, various methods were applied to identify the genetic alterations, due to issues with tissue availability and sample quality, particularly in the NGS-failed FFPE tissue samples. Although the use of FISH, IHC, and direct sequencing is beneficial for detecting *RET* or *ALK* fusions and the *DICER1* variant (Supplemental Table 1), there may have been unidentified genetic drivers in the NGS-failed samples (n = 15), leading to underestimation of the detection rate in our study. Nonetheless, this is the first pediatric study to show that fusion-targeted therapy reactivated radioiodine uptake and inhibited tumor growth in ^{131}I-refractory PTC patients. In addition, we performed an *in vitro* experiment to demonstrate the role of fusion-targeted therapy in restoring NIS expression and radioiodine uptake.

In summary, oncogenic fusions are the main genetic drivers of PTCs identified in young children. Selective fusion-targeted therapy may restore radioiodine avidity, as well as produce a tumor response in pediatric fusion oncogene PTC cases with ^{131}I-refractory advanced characteristics, making molecular testing imperative for pediatric patients presenting with advanced PTC.

Methods

Patients and tissue samples

In total, 106 tumor tissue samples from pediatric PTC patients, obtained by Seoul National University Hospital (SNUH) between January 1983 and March 2020, were analyzed (Figure 1). Fresh frozen tumor tissue samples were obtained from 12 pediatric patients aged < 20 years. The transcriptome data of adult patients aged ≥ 20 years (125 cases at SNUH) were analyzed to compare gene expression profiles (11). Detailed information on the treatment and follow-up strategies was obtained (12), and disease outcomes were categorized as no evidence of disease (NED), biochemical disease, or structural disease (persistent or recurrent disease) (32), as described in the Supplemental methods.

Genomic profiling by NGS, direct sequencing, FISH, and/or IHC

The genetic analysis was performed using whole-genome sequencing (WGS), targeted sequencing, RNA sequencing, direct sequencing, FISH, and/or IHC according to the tissue availability (Figure 1, Supplemental Table 1). RNA sequencing libraries of fresh frozen and FFPE tissues were constructed using the TruSeq RNA Library Preparation Kit v2 and TruSeq RNA Access Library Preparation Kit (Illumina, San Diego, CA, USA), respectively. The library constructed for WGS used the Illumina TruSeq Nano DNA Preparation Kit (Illumina). The subsequent NGS analysis is described in the Supplemental methods. The *BRAF* exon 15, *TERT* promoter (C228T and C250T) region, *H/K/NRAS* codons 12, 13, and 61; and sequence encoding the *DICER1* RNase IIIb domain were amplified by PCR using appropriate primers to directly sequence the *BRAF, RAS, DICER1*, and *TERT* genes (Supplemental Table 8) (18). The FISH probe for the *NTRK* and *RET* rearrangements, and the antibodies for IHC of $BRAF^{V600E}$, *NRAS* Q61R, *ALK*, and *pan-Trk*, are described in the Supplemental methods.

Cell culture and in vitro assays

The human *TPR-NTRK1* expression vector was constructed by subcloning the corresponding cDNAs into the pcDNA6/V5-His A expression vector (Thermo Fisher Scientific, Waltham, MA, USA) (Supplemental Figure 5, Supplemental Table 5). The pcDNA6/V5-His A-TPR-NTRK1 fusion construct $Nthy^{TPR\text{-}NTRK}$, and the unmodified vector control, pcDNA6/V5-His A ($Nthy^{WT}$), were transfected into N-thyroid cells (ECACC, Salisbury, UK). The degree of overexpression of $Nthy^{TPR\text{-}NTRK}$ cells was similar to that seen in NTRK fusion cancer, based on comparison of the NTRK mRNA levels among the $Nthy^{WT}$, normal thyroid tissue, $Nthy^{TPR\text{-}NTRK}$ cells, and thyroid cancer tissues with a TPR-NTRK fusion (Supplemental Figure 6).

After incubation with larotrectinib (kindly provided by Bayer AG), expression of mRNA and protein, and ^{125}I uptake, were analyzed; ^{131}I clonogenic assays were performed in $Nthy^{TPR\text{-}NTRK}$ or $Nthy^{WT}$ transfected cells, as described previously (33). NIS mRNA and protein expression was analyzed by RT-PCR (using the appropriate primers; Supplemental Table 8) and immunoblotting (with an anti-NIS antibody; Thermo Fisher Scientific), respectively.

Statistics

All analyses were performed using SPSS for Windows statistical software (version 25.0; SPSS Inc., Chicago, IL, USA). Differences in continuous variables were compared between two groups using Student's *t*-test or the Mann–Whitney *U* test. Categorical variables were compared between the two groups using the chi-square test or Fisher's exact test, while the chi-square test for trend or logistic regression was used for comparisons among three groups. Recurrence-free survival plots were constructed using the Kaplan-Meier method, and groups were compared using the Cox proportional hazard model. The hazard ratios (HRs), 95% confidence intervals (CIs), and P-values are reported. A P-value < 0.05 was considered significant.

Data availability

The RNA sequencing data set produced in this study was deposited in the NCBI's Sequence Read Archive (SRA PRJNA701374).

Study approval

Written informed consents were obtained in line with the institutional review board of the SNUH (approved ID: H-1307-034-501, 1505-023-670).

Author contributions

YAL, J-IK, and YJP conceived and designed the study. HL, S-WI, and JC analyzed NGS data under the supervision of J-IK. YAL, D-YO, HJK, J-KW, KCJ, DK, E-JC, HJH, JCP, J-HK, CHS, and YJP delivered multidisciplinary therapy to pediatric thyroid cancer patients, prepared tissue samples, explored the pathologies in play, or collected imaging data. In particular, D-YO, and HJK delivered fusion-directed targeted therapy. O-KK, JMO, and B-CA performed the *in vitro* experiment. YAL, HL, S-WI, YSS, J-IK, and YJP drafted the paper. LJW contributed to data interpretation, and manuscript revision. All authors contributed to multiple revisions and approved the final manuscript.

Acknowledgements

The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. Two patients with progressive ^{131}I-refractory lung metastases harboring a *TPR-NTRK1* or *CCDC6-RET* fusion received fusion-targeted therapy after obtaining the each informed consent; Larotrectinib (NCT02576431; NAVIGATE) and Selpercatinib (#LOXO-RET-18018), respectively. The Bayer AG and Eli Lilly and Company reviewed the report and provided comments for the authors to consider. All this research except the clinical trials for the 2 patients was supported by the Basic Science Research Program through the National Research Foundation of Korea,

funded by the Ministry of Science, ICT & Future Planning (grant number NRF-2016R1A2B4012417 and 2019R1A2C2084332), a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number H14C1277) and Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (grant number 2020R1A6A1A03047972). This study was also supported by the Seoul National University Hospital Research Fund (grant number 04-2015-0830). The authors thank Nicoletta Brega (Bayer AG) and Eli Lilly and Company for medical monitoring of the patient treated with larotrectinib and selpercatinib, respectively. The authors especially thank Jeong Seon Lee, Hwan Hee Kim, Gyeongseo Jung, and Sookyung Kim for sample preparation and molecular analyses.

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Figure legends

Figure 1. Genetic analysis of PTC samples. One hundred six tumor tissue samples of pediatric patients with PTC (22 males and 84 females, median age 14.3 years, ranges 4.3 to 19.8 years) were analyzed to profile genetic alterations using whole genome sequencing (WGS), targeted sequencing, mRNA sequencing, direct sequencing, fluorescence in situ hybridization (FISH), and/or immunohistochemistry (IHC) according to the availability of each tissue.

Figure 2. Age-associated genetic profiles of pediatric PTCs and comparison of the clinicopathological presentation and disease outcomes between fusion oncogene and $BRAF^{V600E}$ PTCs. Age-associated proportions of fusion oncogenes and point mutations, and genetic drivers among the pediatric patients in this study [A and B; aged < 10 years (n = 14), 10–14 years (n = 40), and 15–19 years (n = 52)], and a pooled analysis of 1,704 patients aged < 23 years [C and D; aged < 10 years (n = 68) and 10–22 years (n = 468), plus other cases without detailed age information]. Comparison of the clinicopathological presentation (E), and disease outcomes (F) among three pediatric groups [fusion < 10 years (n = 13), fusion 10–19 years (n = 18), and $BRAF^{V600E}$ PTCs (all 10–19 years, n = 41)]. Comparison of the clinicopathological presentation (G) and disease outcomes (H) between the pediatric fusion (n = 31) and adult fusion (n = 12) groups, and between the pediatric $BRAF^{V600E}$ (n = 41) and adult $BRAF^{V600E}$ (n = 68) groups. Categorical variables were compared between the two groups using the chi-square test or Fisher's exact test, while the chi-square test for trend or logistic regression was used for comparisons among three groups (*, $p < 0.05$; **, $p < 0.01$; ***, and $p < 0.001$). Recurrence-free survival (I) was compared among these four groups with reference to the pediatric fusion group. Recurrence-free survival plots were constructed using the Kaplan-Meier method, and groups were compared using the Cox proportional hazard model. The hazard ratios (HRs), 95% confidence intervals (CIs), and P-values are reported. ETE, extrathyroidal extension; LN, lymph node; NED, no evidence of disease; BCD, biochemical disease; SD, structural disease

Figure 3. Comparison of expression signatures between pediatric and adult PTCs. A and B show the results of K-means clustering (obtained via principal component analysis). (A) Comparison between 12

pediatric PTCs (9 fusion oncogenes and 3 *BRAF*^{V600E}-PTCs) and 125 adult PTCs, including BRAF-like, RAS-like and NBNR. (B) Comparison between pediatric (n = 9) and adult (n = 12) PTCs with fusion oncogenes. The ages and mutation types are represented by shape and color, respectively. (C) The ERK score, thyroid differentiation score (TDS), and SLC5A5 (sodium-iodide symporter, NIS) analysis results are represented by box plots (left). The results of the TDS–ERK score analysis are displayed as a scatterplot (right). (D) The heatmap shows the expression levels of 16 TDS genes associated with thyroid function and metabolism. Comparison of TDS genes between the pediatric and adult fusion groups, and between the pediatric and adult *BRAF*^{V600E} groups using fresh-frozen tissue samples, presented age within each group. (E) Comparison of TDS genes between pediatric PTCs and normal thyroid tissues based on analysis of FFPE samples. Two young girls (P1 and P8) with progressive ^{131}I-refractory lung metastasis had low expression of *SCL5A5* in their tumor tissues.

Figure 4. Selective fusion-targeted therapy decreased the tumor extent and restored radioiodine uptake in ^{131}I-refractory progressive metastatic pediatric PTCs*;* a 4.3-year-old girl with *TPR-NTRK1* fusion-positive PTC (A and B), and a 7.4-year-old girl with *CCDC6-RET* fusion-positive PTC (C and D). (A) The post-treatment WBS showed remnant thyroid uptake only. Radioiodine uptake was restored in cervical LN and lung lesions after 12 weeks of larotrectinib therapy. (B) CT revealed a dramatic improvement in the LN and lung target lesions (decreased to 35% of baseline) after 4 weeks. The patient achieved complete remission after 21 months and remained responsive, with no dose-limiting toxicity seen during 41 months of larotrectinib therapy. (C) The post-treatment WBS revealed minimal lung uptake. Radioiodine uptake was restored in the entire lung field after 5 months of selpercatinib therapy. The addition of ^{131}I of 60 mCi at 13 months after starting selpercatinib led to remarkable radioiodine uptake in the lung field. (D) Lung lesions were markedly improved according to a chest radiograph obtained after 10 days, and decreased to 42.9% of baseline on a CT scan after 4 weeks. The patient achieved partial remission after 4 weeks and remained responsive, with no dose-limiting toxicity seen during 29 months of selpercatinib therapy.

Figure 5. *In vitro* effects of larotrectinib on radioiodine uptake capacity and cell growth. (A) Baseline ^{125}I uptake decreased in $Nthy^{TPR\text{-}NTRK}$ cells compared to $Nthy^{WT}$ cells, but was restored by larotrectinib treatment mediated by NIS; this was demonstrated by blocking the effects with potassium perchlorate ($KClO_4$). Expression of NIS at the mRNA (B) and protein (C) levels tended to increase in $Nthy^{TPR\text{-}NTRK}$ cells with larotrectinib (50 μM) treatment. (D) The colony-forming ability of $Nthy^{TPR\text{-}NTRK}$ cells did not change after ^{131}I therapy alone but decreased after larotrectinib treatment, and then further decreased after combined ^{131}I and larotrectinib therapy. LAR, larotrectinib (50 μM); NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, and $p < 0.001$ using Student's *t*-test or One-way ANOVA with Bonferroni's multiple-comparison test. All data are mean ±SD.

588 **Table 1.** Comparison of clinicopathological characteristics between pediatric PTC patients harboring the fusion oncogene and *BRAF*^{V600E}

	N of total	Total patients (n = 106)	N of fusion	Fusion (n = 31)	N of *BRAF*^{V600E}	*BRAF*^{V600E} (n= 41)	*P*-value (fusion vs. *BRAF*^{V600E})
Age (years)	106	14.3 ± 3.8	31	11.1 ± 4.2	41	16.3 ± 2.3	<0.001
Age group (<10/ 10-14/ 15-19 years), n (%)	106	14/40/52 (13.2/37.7/49.1)	31	13/11/7 (41.9/35.5/22.6)	41	0/11/30 (0/26.8/73.2)	<0.001
Sex (males/ females), n (%)	106	22/84 (20.8/79.2)	31	6/25 (19.4/ 80.6)	41	6/35 (14.6/85.4)	0.751
Previous history of radiotherapy (yes/ no), n (%)	106	9/97 (8.5/91.5)	31	3/28 (9.7/90.3)	41	1/40 (2.4/97.6)	0.308
Thyroidectomy (total thyroidectomy/ lobectomy)	106	97/9 (91.5/8.5)	31	30/1 (96.8/3.2)	41	38/3 (92.7/7.3)	0.629
LN dissection, total (yes/ no), n (%)	105	83/22 (79.3/20.9)	31	28/3 (90.3/9.7)	40	33/7 (82.5/17.5)	0.496
Lateral LN dissection (yes/ no), n (%)	102	46/56 (45.1/54.9)	31	22/9 (71.0/29.0)	37	11/26 (29.7/70.3)	0.001
Radioiodine therapy (yes/ no), n (%)	102	71/31 (69.6/30.4)	31	27/4 (87.1/12.9)	40	23/17 (57.5/42.5)	0.009
PTC subtype (classic variant/ diffuse sclerosing variant/ other subtypes [b]), n (%)	104	75[a]/14/15[b] (72.1/13.5/14.4)	31	16/13/2 (51.6/41.9/6.5)	39	35/0/4 (89.7/0/10.3)	0.025
Size (cm)	102	2.1 ± 1.3	31	2.8 ± 1.5	38	1.4 ± 1.0	<0.001
Size (> 2cm/ ≤ 2cm), n (%)	102	46/56 (45.1/54.9)	31	20/11 (64.5/35.5)	38	10/28 (26.3/73.7)	0.002
Multifocality (yes/ no), n (%)	105	40/65 (38.1/61.9)	31	14/17 (45.2/43.8)	40	13/27 (32.5/67.5)	0.329
Extrathyroidal extension (yes/ no), n (%)	100	70/30 (70.0/30.0)	31	26/5 (83.9/16.1)	38	23/15 (60.5/39.5)	0.028

No/ minimal/ gross, n (%)	100	30/45/24 (30.0/45.0/25.0)	31	4/17/9 (13.3/56.7/30.0)	38	15/17/6 (39.5/44.7/15.8)	0.020
LN metastasis (yes/ no), n (%)	101	74/27 (73.3/26.7)	31	29/2 (93.5/6.5)	38	26/12 (68.4/31.6)	0.015
Lateral LN metastasis (yes/ no), n (%)	97	24/73 (24.7/75.3)	31	11/20 (35.5/64.5)	35	6/29 (17.1/82.9)	0.101
Lung metastasis (yes/ no), n (%)	103	20/83 (19.4/80.6)	31	13/18 (41.9/58.1)	40	1/39 (2.5/97.5)	<0.001
Follow-up years, median (range)	106	7.3 (0.3-37.3)	31	4.8 (0.9-34.3)	41	8.0 (0.6-37.3)	0.106
Disease outcome at any event (NED/ BCD/ SD)[c], n (%)	97	53/10/34 (54.6/10.3/35.1)	29	6/6/17 (20.7/20.7/58.6)	37	27/4/6 (73.0/10.8/16.2)	<0.001
Disease outcome at last follow-up (NED/ BCD/ SD)[c], n (%)	97	60/15/22 (61.9/15.5/22.7)	29	8/6/15 (27.6/20.7/51.7)	37	29/7/1 (78.4/18.9/2.7)	<0.001

589 Data are expressed as mean ± standard deviation (mean ± SD) or number (%).

590 [a]Two of 75 patients had multifocal PTCs harboring different subtype (classic variant and follicular variant, and classic variant and solid variant, respectively)

591 [b]Other subtypes include 9 follicular variant, 2 solid variant, 1 tall cell variant, and 1 Hobnail variant.

592 [c]Disease outcomes were categorized as no evidence of disease (NED), biochemical disease (BCD), and structural disease (SD, persistence or recurrence). NED, no evidence of

593 disease, defined as the absence of structural abnormalities on imaging and undetectable serum thyroglobulin levels (suppressed or stimulated) for 12 months or longer until the

594 last follow-up; SD, structural disease, defined as the presence of structural abnormalities; BCD, biochemical disease, defined as detectable suppressed or stimulated thyroglobulin

595 levels in the absence of structural abnormalities on imaging modalities. Stable and progressive were defined according to the RECIST criteria

596

597 **Table 2.** Clinicopathological presentation and disease outcomes in pediatric PTC patients harboring a fusion oncogene

ID	Age (yrs)	Sex	Sporadic or radiotherapy[a]	PTC Subtype	Genetic alteration	Size (cm)	Multi-focality	ETE	LN meta	Distant meta	FU years	Disease outcome[c] (any event)	Disease outcome[c] (at last follow-up)
1	4·3	F	Sporadic	cPTC	*TPR-NTRK1*	3·6	No	Minimal	Yes	Lung	5.3	SD (Persist, LN & lung)	SD (Progress, LN & lung)
2	5·2	F	Sporadic	FVPTC, infiltrative	*ETV6-NTRK3*	2·1	No	Minimal	Yes	No	5.0	BCD	BCD
3	10.4	F	Sporadic	FVPTC, infiltrative	*ETV6-NTRK3*	1.5	No	N/A	Yes	Yes	2.2	SD (Persist, LN & lung)	SD (Persist, lung)
4	14.2	F	Sporadic	cPTC	*TPM3-NTRK1*	2.1	No	No	Yes	No	0.6	Ongoing	Ongoing
5	5·1	F	Sporadic	cPTC	*VCL-RET*	2·5	No	Gross	Yes	Lung	8.2	NED	NED
6	6·4	M	Sporadic	DSV-PTC	*NCOA4-RET*	1·3	Yes	Minimal	Yes	No	7.2	SD (Recur)	NED
7	7·1	F	Sporadic	DSV-PTC	*TRIM24-RET*	1·5	No	No	Yes	Lung	8.8	SD (Persist, lung)	SD (Persist, lung)
8	7·4	F	Sporadic	DSV-PTC	*CCDC6-RET*	2·8	No	Minimal	Yes	Lung	3.5	SD (Persist, LN & lung)	SD (Progress, LN & lung)
9	7·6	F	Sporadic	cPTC	*RET-NCOA4*	1·0	No	Gross	Yes	No	34.3	SD (Recur)	NED
10	7·8	M	Radiotherapy[a]	cPTC	*NCOA4-RET*	2·6	Yes	Gross	Yes	No	7.0	BCD	BCD
11	9.0	M	Sporadic	DSV-PTC	*ERC1-RET*	7.0	Yes	Minimal	Yes	Lung	3.4	SD (Persist, lung)	SD (Progress, lung)
12	9·6	M	Sporadic	cPTC	*TRIM24-RET*	2·4	No	Minimal	Yes	No	4.6	SD (Recur)	BCD
13	9·9	M	Sporadic	DSV-PTC	*NCOA4-RET*	1·6	No	Minimal	Yes	No	7.2	BCD	BCD
14	10·1	F	Sporadic	cPTC	*NCOA4-RET*	2·2	Yes	Gross	Yes	Lung	9.0	SD (Persist, lung)	SD (Persist, lung)
15	10·3	F	Sporadic	DSV-PTC	*CCDC6-RET*	2·5	Yes	Gross	Yes	Lung	9.5	SD (Persist, LN & lung)	SD (Persist, lung)
16	10.4	F	Sporadic	DSV-PTC	*NCOA4-RET*	4.1	Yes	Gross	Yes	No	2.5	SD (Recur)	SD (Recur)

17	10·5	F	Sporadic	DSV-PTC	*CCDC6-RET*	7·0	No	Minimal	Yes	No	1.7	SD (Recur)	SD (Recur)
18	13·3	F	Radiotherapy[a]	cPTC	*CCDC6-RET*	1·7	No	Minimal	No	No	4.8	NED	NED
19	13·7	F	Sporadic	DSV-PTC	*ANK3-RET*	3·5	No	Minimal	Yes	No	2.0	NED	NED
20	14·3	F	Sporadic	DSV-PTC	*KTN1-RET*	5·0	No	Gross	Yes	Lung	2.7	SD (Persist, lung)	SD (Persist, lung)
21	14·5	F	Sporadic	cPTC	*CCDC6-RET*	4·2	No	No	Yes	No	11.7	NED	NED
22	16·1	M	Sporadic	cPTC	*CCDC6-RET*	1·9	Yes	Minimal	Yes	No	10.7	BCD	BCD
23	16·1	F	Sporadic	DSV-PTC	*CCDC6-RET*	2·3	Yes	Minimal	Yes	Lung	3.1	SD (Persist, lung)	SD (Persist, lung)
24	17·2	F	Sporadic	cPTC	*CCDC6-RET*	0·9	No	No	No	No	13.2	NED	NED
25	18·9	F	Sporadic	DSV-PTC	*CCDC6-RET*	1·9	Yes	Minimal	Yes	No	3.2	BCD	BCD
26	4·5	F	Sporadic	DSV-PTC	*STRN-ALK*	3·3	Yes	Minimal	Yes	Lung	1.6	Ongoing	Ongoing
27	8·9	F	Sporadic	cPTC	*EML4-ALK*	1·4	Yes	Minimal	Yes	No	3.4	NED	NED
28	12·1	F	Sporadic	cPTC	*ALK*[b]	1·8	No	Minimal	Yes	Lung	8.8	SD (Persist, lung)	SD (Persist, lung)
29	15·6	F	Sporadic	cPTC	*RBMS3-ALK*	4·0	Yes	Gross	Yes	Lung	15.3	SD (Persist, lung)	SD (Persist, lung)
30	15·9	F	Sporadic	cPTC	*ALK*[b]	4·0	Yes	Gross	Yes	No	2.0	BCD	BCD
31	18·1	F	Sporadic	cPTC	*ALK*[b]	2·2	Yes	Gross	Yes	No	4.1	SD (Persist, LN)	BCD

598 ETE, extrathyroidal extension; LN, lymph node; meta, metastasis; FU, follow-up; cPTC, classic variant PTC; FVPTC, follicular variant PTC; DSV-PTC, diffuse sclerosing

599 variant PTC; NED, no evidence disease; BCD, biochemical disease; and SD, structural disease.

600 [a]Childhood cancer survivors who had received radiotherapy

601 [b]Fusions where no 5′ partner specified

[c]Disease outcomes were categorized as no evidence of disease (NED), biochemical disease (BCD), and structural disease (SD, persistent [Persist] or recurrent [Recur]). NED, no evidence of disease, defined as the absence of structural abnormalities on imaging and undetectable serum thyroglobulin levels (TSH-suppressed or stimulated) for 12 months or longer until the last follow-up; SD, structural disease, defined as the presence of structural abnormalities showing locally-advanced and/or metastastic disease; BCD, biochemical disease, defined as detectable serum thyroglobulin levels (TSH-suppressed or stimulated) in the absence of structural abnormalities on imaging modalities. Stable and progressive disease were defined according to Response Evaluation Criteria in Solid Tumors criteria.